Annals of Hepatology
The Official Journal of the Mexican Association of Hepatology, the Latin-American Association for Study of the Liver and the Canadian Association for the Study of the Liver

ORIGINAL ARTICLE

Long-Term, Low-Dose Exposure to Microcystin-LR Does not Cause or Increase the Severity of Liver Disease in Rodents

Meaghan Labine,* Yuewen Gong,** Gerald Y. Minuk*

* Department of Pharmacology and Therapeutics, Faculties of Medicine. University of Manitoba, Winnipeg, Manitoba, Canada.
** Pharmacy. University of Manitoba, Winnipeg, Manitoba, Canada.

ABSTRACT

Background. Acute exposure to high concentrations of microcystin-LR (MC-LR) can cause significant hepatocyte injury. Aim. To document the effects of long-term, low-dose MC-LR exposure on hepatic inflammation and fibrosis in mice with healthy and diseased livers. **Material and methods.** Male CD1 mice (N = 20/group) were exposed to 1.0 μg/L of MC-LR in drinking water; 1.0 μg/L MC-LR plus 300 mg/L of the hepatotoxin thioacetamide (MC-LR/TAA); or 300 mg/L TAA alone for 28 weeks. Liver biochemistry and histology were documented at the end of the study period. In addition, hepatic stellate cells (HSCs), were exposed *in vitro* to MC-LR (0.1-10,000 μg/L) and monitored for changes in cell metabolism, proliferation and activation. **Results.** Liver biochemistry and histology were essentially normal in MC-LR alone exposed mice. MC-LR/TAA and TAA alone exposed mice had significant hepatic inflammation and fibrosis but the extent of the changes were similar in the two groups. *In vitro*, MC-LR had no effect on HSC metabolism, proliferation or activation. **Conclusion.** Long-term, low-dose exposure to MC-LR is unlikely to lead to chronic liver disease in the setting of a normal liver or exacerbate existing liver disease in the setting of ongoing hepatitis.

Key words. Cyanobacteria. Blue-green algae. Cyanotoxins. Microcystins. Hepatic fibrosis. Cirrhosis. Chronic liver disease. Hepatic stellate cells.

INTRODUCTION

Cyanobacteria (blue-green algae) contamination of drinking water is an increasingly common public health concern throughout the world.[1,2] Previous case reports and animal studies have documented that acute exposure to various cyanobacterial toxins (cyanotoxins) and microcystins (MC) in particular, can cause severe hepatocyte injury and liver failure.[3-5] Based on these findings, the World Health Organization (WHO) designated 1.0 μg/L of MC-LR as the maximum safe concentration of cyanotoxin permissible in human drinking water.[6]

The mechanism whereby cyanotoxins induce hepatocyte injury is unclear but appears to involve increased oxidative stress, inhibition of serine/threonine phosphatase activity and/or activation of Akt and p38/ERK/JNK signaling.[7-11] There are also data to suggest that sub-lethal exposures to cyanotoxins enhance hepatic lipotoxicity and are fibrogenic in the liver and possibly the heart by activating tissue myofibroblasts.[11-15]

In the present study we documented the effects of long-term, low-dose exposure to MC-LR in mice with healthy livers and those with thioacetamide (TAA)-induced liver injury. We also documented the *in vitro* effects of a range of MC-LR concentrations on the metabolic activity, proliferation and activation of hepatic stellate cells (HSC), the myofibroblasts of the liver principally responsible for hepatic fibrogenesis.

MATERIAL AND METHODS

Adult male CD1 mice weighing 20-24 g, obtained from the University of Manitoba Animal Care Facility, were acclimatized for two weeks prior to use. Animals had free access to commercial laboratory chow and

Manuscript received: December 15, 2016. **Manuscript accepted:** April 16, 2017.

DOI:10.5604/01.3001.0010.5288

were maintained on a 12 h light:dark cycle throughout the study.

Four study groups (N = 20/group) were analyzed: those allowed free access to water alone, water containing 1.0 μg/L of MC-LR (Sigma Aldrich, Oakville, ON, Canada), 1.0 μg/L of MC-LR and 300 mg/L of TAA (Sigma Aldrich) or 300 mg/L of TAA alone. Following 28 weeks of exposure, mice were euthanized, body/liver weights recorded, blood tested by standard biochemical techniques for evidence of hepatic inflammation (serum alanine aminotransferase - ALT) and dysfunction (serum total bilirubin - TB) and hepatic histology was examined. The latter consisted of staining 5 μm slices of paraffin embedded tissue with Hemotoxylin and Eosin (H&E) and Picric Acid Sirius Red for evidence of inflammatory activity and fibrosis respectively. H&E slides were graded for inflammation according to the metavir inflammation scoring scale using the following units; 0 = no inflammation, 1 = minimal inflammation/occasional spotty necrosis, 2 = mild inflammation/little hepatocellular damage, 3 = moderate inflammation with noticeable hepatocellular damage and 4 = severe inflammation with prominent diffuse hepatocellular damage. Picric Acid and Sirius Red stained slides were graded for fibrosis according to the metavir fibrosis grade scale using the following units; 0 = no scarring, 1 = fibrosis confined to the portal tracts, 2 = fibrosis extending beyond the portal tracts, 3 = bridging fibrosis; fibrosis spreading and connecting to central veins or other portal tracts and 4 = cirrhosis or advanced scarring of the liver.

In vitro studies were performed with a rodent hepatic stellate cell (HSC) line (CFSC-2G), a gift from Dr. Y. Gong, Faculty of Pharmacy, University of Manitoba. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin and 100 μg/mL streptomycin and incubated at 37 °C (in a humidifier in a 5% $C0_2$ atmosphere) until tested for oxidative stress, serine/threonine phosphatase activity, proliferation and activation (transition to a myofibroblast phenotype).

Oxidative Stress

CFSC-2G cells were plated in 96 well plates at a density of 10,000 cells per well in 100 μ/L of supplemented DMEM medium and allowed to adhere overnight. Medium was replaced with medium containing various concentrations of MC-LR (0.1-10,000 μg/L), 0.1 μg/L of okadaic acid (OA) (positive control) or medium alone. After 24 h of incubation at 37 °C, CellRox Deep Red reagent oxidative stress dye (Life Technologies, Grand Island, NY, USA) was added to each well at a concentration of 5 μM and incubated at 37 °C for 30 min according to manufacturer's instructions. Cells were then washed x3 using sterile PBS. Readings were taken using a Ziess Axio observer Z1 fluorescent microscope at a 40x objective against untreated cells containing PBS and 5 μM of CellRox dye and analyzed using Ziess AxioVision 4 software.

Serine/Threonine Phosphatase Inhibition

CFSC-2G cells were seeded at 200,000 cells/well in DMEM supplemented medium in 6 well plates and allowed to attach overnight. Medium containing various concentrations of MC-LR (0.1- 10,000 μg/L), okadaic acid (positive control) or medium alone was then added. Cells were allowed to incubate for an additional 24 hrs, washed with sterile PBS, and lysed using radioimmunoprecipitation (RIPA) lysis buffer plus protease inhibitor. Cell lysates were then added to a RediPlate 96 EnzChek Serine/threonine Phosphatase Assay Kit Plate (Molecular Probes, Eugene, OR, United States) as per the manufacturer's instructions. Fluorescence readings were measured using a BioTek Synergy 4 microplate reader (BioTek Instruments, Winoosky, VT, USA) at an excitation/emission maxima of 358/452.

Cellular Proliferation

WST-1[2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] (WST-1) (Roche Applied Science, Laval, QC, Canada) was employed to document CFSC-2G proliferative activity. Briefly, 96 well plates were seeded with CFSC-2G cells at a density of 2,000 cells/well in DMEM medium and allowed to attach for 4 h. Medium was then replaced with medium containing various concentrations of MC-LR (0.1-1,000 μg/L), Epidermal Growth Factor (100 μg/L) or Platelet Derived Growth Factor (100 μg/L) (positive controls). Following 24, 48, 72 and 96 h incubation at 37 °C, cells were washed with PBS and fresh medium containing 10 μ/L of the WST-1 reagent was added to each well. Cells were then incubated for 2 h at 37 °C, and absorbance read at 450 nm (reference wavelength 630 nm) using a BioTek plate reader (BioTek Instruments). Readings were compared against cell free blanks containing medium alone and WST-1 reagent.

Activation

Activation of HSCs to a myofibroblast phenotype was determined by documenting the expression of

smooth muscle actin alpha (SMAA) protein. Following exposure to MC-LR or positive controls as described above for cell proliferation, CFSC-2G cells were lysed using RIPA lysis buffer plus protease inhibitor. Protein levels were quantified using a Bradford Protein Assay Kit as per the manufacturer's instructions (Bio-Rad Laboratories, Mississauga, ON, Canada). Readings were taken at an absorbance of 560 nm (reference wavelength optical density 630 nm) using a BioTek plate reader (BioTek Instruments). Aliquots were electrophoresed through 10% polyacrylamide-SDS gels and resolved proteins transferred to Nitro-pins 2,000 membranes (Micron Separations, Westborough, MA, USA). Membranes were blocked with 5.0% skim milk in tris-buffered saline for 1 h at room temperature, incubated overnight at 4 ºC in the presence of 1:1000 anti-SMAA (Roche Molecular Biochemicals, Laval, QC, Canada), diluted in PBS + 1% Tween-20. Membranes were then washed in PBS + 1% Tween-20 5x, exposed to peroxidase-conjugated sheep anti-mouse secondary antibody (1:1000 diluted in PBS + 1% Tween-20) (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at room temperature and washed in PBS +1% Tween 20 5x. Bands were detected using an enhanced chemiluminescence system (Amersham-Pharmacia Biotech, Piscataway, NJ, USA).

STATISTICS

Body/liver weights and plasma chemistry comparisons were performed with Sigma Plot software applying one-way anova analysis. Liver histology results for inflammation and fibrosis were analyzed for significance using the Kruskal-Wallis one-way anova on ranks method, comparing the treatment groups to negative controls. A two-sided $P < 0.05$ was considered significant.

RESULTS

Table 1 provides the results of body and liver weight determinations at the time of sacrifice. Compared to water alone exposed mice, MC-LR exposed mice had similar body and liver weights. However, MC-LR/TAA

Table 1. Body Weight, Liver Weight and Percent Liver to Body Wight in Mice Exposed to Water Alone (control), MC-LR Alone, MC-LR plus TAA or TAA Alone for 28 Weeks.

Treament group	BWT	LWT	LWT % BWT
Control	53.8 ± 5.3 g	2.8 ± 0.3 g	5.0 ± 0.1%
MC-LR	55.8 ± 5.2 g	2.7 ± 0.6 g	4.8 ± 0.1%
MC-LR + TAA	45.7 ± 4.1 g*	3.2 ± 0.6 g	6.0 ± 0.1%**
TAA	47.4 ± 3.5 g*	3.1 ± 0.6 g	6.5 ± 0.2%**

** P < 0.05, ** P < 0.01, BWT: Body weight. LWT: Liver weight. MC-LR: Microcystin-LR. TAA: Thioa cetamide.*

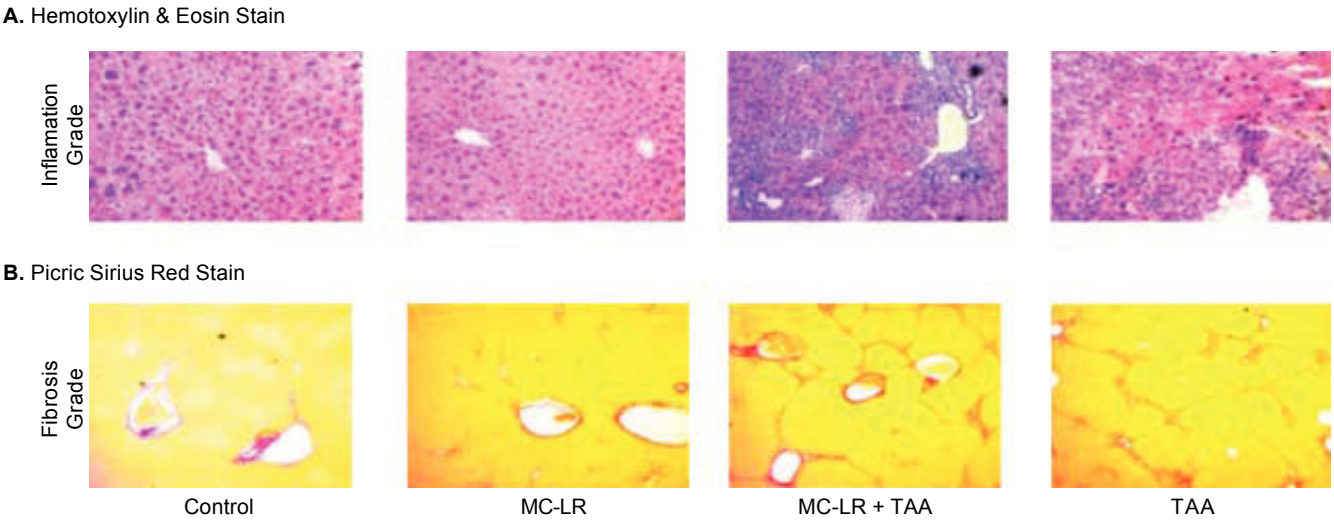

Figure 1. *Hematoxylin & Eosin and Picric Cirius stains of livers of male CD1 mice exposed to water alone (control) microcystin-LR (MC-LR) alone, MC-LR plus thioacetamide (TAA) or TAA alone for 28 weeks.*

and TAA alone exposed groups had significantly lower body weights and higher liver/body weight ratios when compared to water alone exposed controls ($p < 0.01$ respectively).

Serum ALT and TB determinations were also measured at the time of sacrifice. Levels were similar in water and MC-LR alone exposed mice but significantly elevated in the MC-LR/TAA and TAA alone exposed cohorts ($p < 0.05$). The extent of the elevations in ALT and TB values were similar in the latter two cohorts.

The results of H&E staining for hepatic inflammation are provided in figure 1 and semi-quantitatively in table 2. There was either no or minimal evidence of hepatic inflammatory activity in the livers of water and MC-LR alone exposed mice while inflammation was significantly increased (but to a similar extent) in the MC-LR/TAA and TAA alone cohorts (METAVIR scores: 2.6 ± 1.0 and 3.3 ± 0.7 respectively) when compared to water alone controls ($p < 0.05$ respectively).

Picric Sirius Red staining for fibrosis (Figure 1 and Table 2) indicated no differences in the extent of fibrosis in livers of water and MC-LR alone exposed mice but significant increases (again, to a similar extent) in the MC-LR/TAA and TAA alone cohorts (METAVIR scores: 2.0 ± 1.0 and 1.8 ± 0.7 respectively) when compared to water alone controls ($p < 0.01$ respectively).

The results of HSC exposure to a range of MC-LR concentrations *in vitro* are provided in figures 2-5. As shown in figures 2 and 3, only at the highest concentrations of MC-LR tested (5,000 and 10,000 μg/L) were significant increases in oxidative stress and inhibition of serine/threonine phosphatase activity observed. Regarding proliferative activity, there were no increases in HSC proliferation after 24, 48 and 96 h of exposure to MC-LR at concentrations as high as 1,000 μg/L compared to buffer alone controls (Figure 4). Similarly, SMAA protein expression remained unaltered following exposure to the same concentrations of MC-LR for identical durations of time (Figure 5).

DISCUSSION

The results of this study indicate that healthy mice exposed to a low concentration of MC-LR in their drinking water for a total of 28 wks (approximately 30% of their anticipated life span) do not develop enlarged

Table 2. Serum Alanine Aminotransferase, Total Bilirubin, Histologic Grade and Stage of Liver Disease in Mice Exposed to Water Alone (control), MC-LR Alone, MC-LR + TAA or TAA Alopne for 28 weeks.

Treatment group	ALT (< 50)	Total bilirubin (0.4)	Inflammation grade (0.4)	Fibrosis grade (0.4)
Control	43.61 ± 17.48	0.11 ± 0.03 mg/dL	0.88 ± 0.89	0.06 ± 0.25
MC-LR	48.35 ± 25.32	0.12 ± 0.05 mg/dL	1.16 ± 0.5	0.26 ± 0.45
MC-LR + TAA	69.12 ± 23.16*	0.22 ± 0.04 mg/dL**	2.57 ± 0.99**	2 ± 1.03**
TAA	68.71 ± 25.71*	0.21 ± 0.03 mg/dL**	3.26 ± 0.65**	1.79 ± 0.71**

** P < 0.05, ** P < 0.01, ALT: Alanine aminotransferase. MC-LR: Microcystin-LR. TAA: Thiocentamide.*

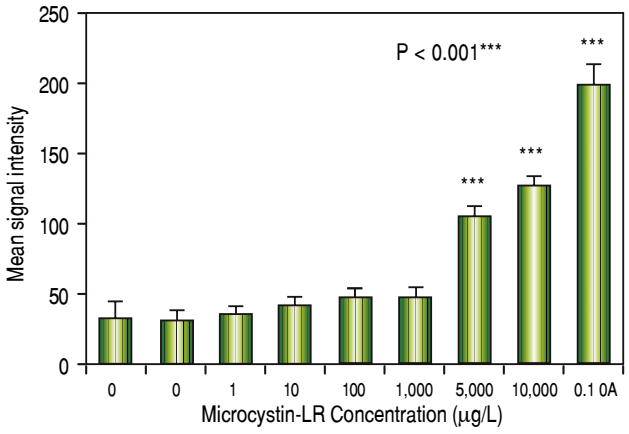

Figure 2. *Oxidative stress of CFSC-2G hepatic stellate cells following 24 hours exposure to varying concentrations of microcystin-LR.*

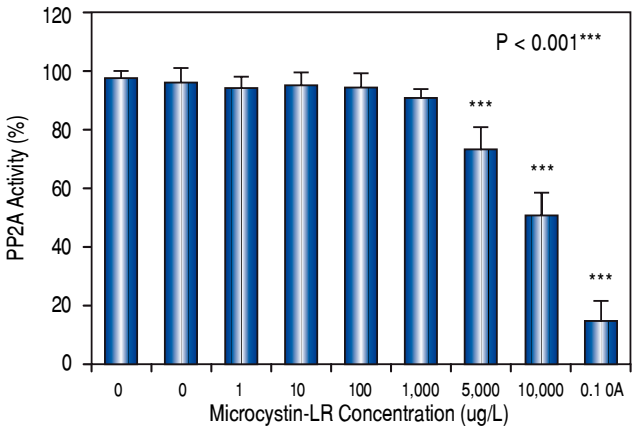

Figure 3. *Serine/threonine phosphatase 2A (PP2A) activity in CFSC-2G hepatic stellate cells following 24 hours exposure to varying concentrations of microcystin-LR.*

livers, biochemical evidence of active hepatic inflammation or dysfunction and have essentially normal liver histology. The results also suggest that long-term, low-dose exposure does not potentiate hepatic inflammation or fibrosis in the setting of active (TAA induced) liver disease. These findings were supported by the results of *in vitro* experiments which demonstrated that low concentrations of MC-LR do not induce oxidative stress, inhibit serine/threonine phosphatase activity, enhance the proliferative activity or activate HSCs to undergo transformation to a myofibroblast phenotype.

Although the above results are reassuring, it must be noted they are not consistent with previous findings from other investigators. Specifically, Elleman, *et al*. described hepatocyte degeneration, scattered lobular necrosis, mononuclear cell infiltration and progressive

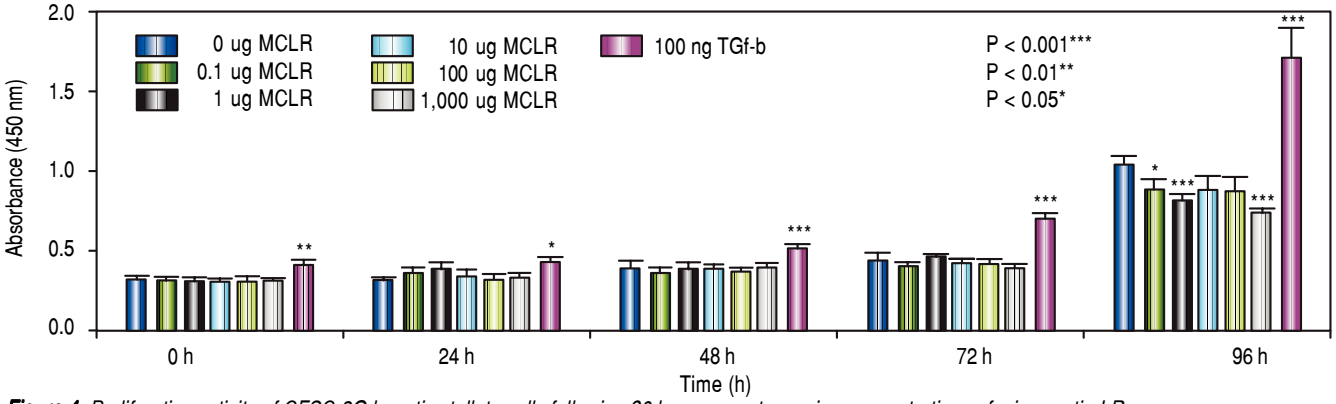

Figure 4. *Proliferative activity of CFSC-2G hepatic stellate cells following 96 h exposure to varying concentrations of microcystin-LR.*

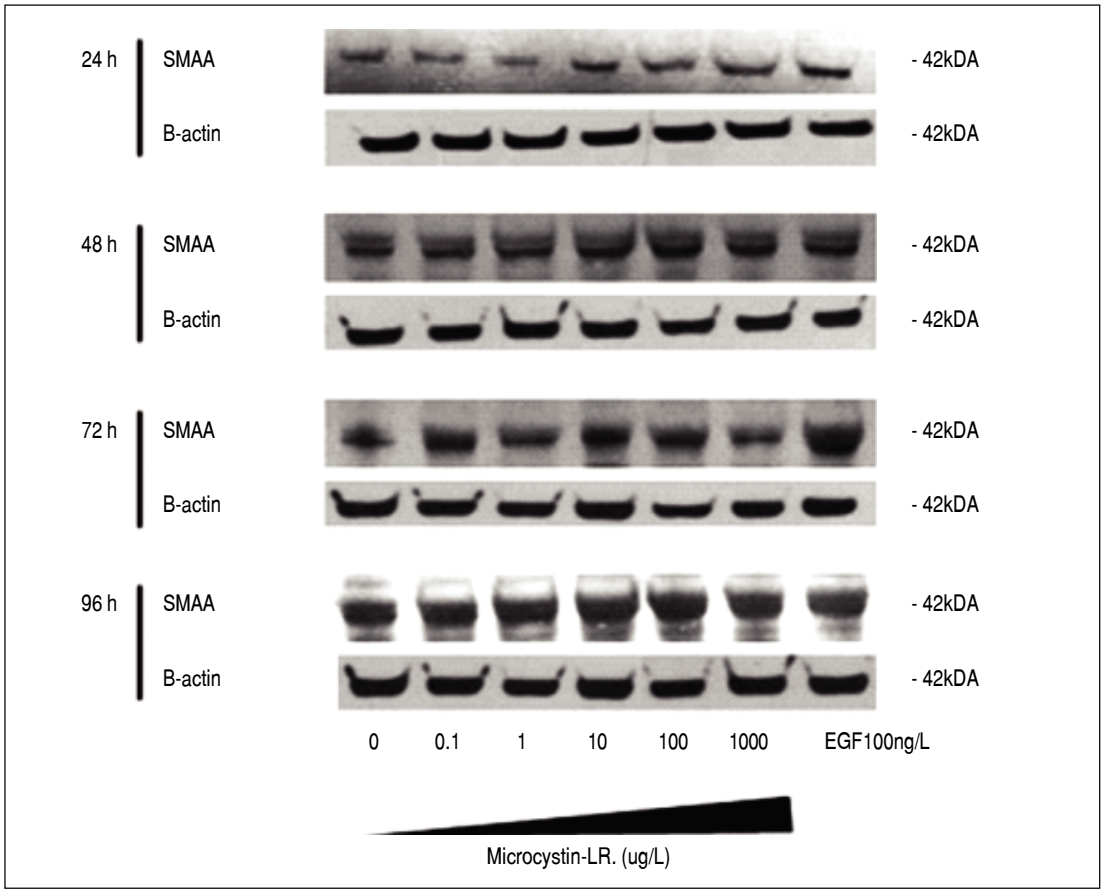

Figure 5. *Smooth muscle alpha actin protein (SMAA) expression in CFSC-2G hepatic stellate cells following 96 h exposure to varying concentrations of microcystin-LR.*

fibrosis in mice treated daily for six weeks with "sublethal" intraperitoneal injections of purified MC-LR at 25%, 50% and 75% of the LD100 dose.[13] In another study, Frangez, *et al*. reported increased peri-portal inflammation and fibrosis in female New Zealand rabbits treated every other day for three weeks with intraperitoneal injections of 7.5 mg/kg cyanobacterial lysates containing 1 mg/g of MC-RR.[14] Finally, He, *et al*. described changes in keeping with nonalcoholic steatohepatitis in BALB/c mice exposed to low-dose (40 μg/kg) MD-LR for 90 days.[11]

The reason(s) for the discrepancy between the above reports and our own findings remain to be determined. Whether differences in species (mice versus rabbits), strains of mice (CD1 versus BALB/c), nutrition (in the above studies MC-exposed mice lost significant amounts of weight) and routes of administration (oral *versus* intraperitoneal) explain the different outcomes requires further research. Also to be considered are differences in the concentrations of MC-LR employed. Unfortunately, the previous studies provided insufficient data to calculate the molarity of MC-LR required for comparative analyses. Finally, it is possible that exposure to more than one MC congener is required to cause inflammation and/or fibrosis, as suggested by Frangez, *et al*.[15]

The need to determine whether cyanotoxins enhance hepatic injury in the setting of existing liver disease was driven by the growing epidemic of obesity and high prevalence of viral hepatitis which together, have resulted in large segments of the general population being diagnosed with chronic liver disease. Thus, whether long-term, low-dose cyanotoxin exposure enhances hepatic inflammation and fibrosis in the setting of existing liver disease is an important clinical question that had yet to be addressed. Here again, the results of the present study are reassuring. Biochemical and histologic evidence of hepatic inflammation and fibrosis were similar in MC-LR/TAA compared to TAA alone exposed mice. Whether the same findings would be obtained in other models of chronic liver disease remains to be determined.

There are a number of limitations to this study that warrant emphasis. First, only the most common and well-studied cyanotoxin, MC-LR, was employed. Second, the amount of water spillage in the animal holding cages was not measured and therefore, precise determinations of MC-LR exposure could not be made. Third, the in vitro studies involved HSCs alone and perhaps co-cultures with hepatocytes or other non-parenchymal cells would have provided different results. Finally, properly designed studies in humans where cyanotoxin contamination of the drinking water has been documented (and quantitated) are required to address the question whether these encouraging findings in rodents can be extrapolated to humans.

In conclusion, the results of the present study do not support concerns that long-term, low-dose exposure to cyanotoxins cause hepatic inflammation or fibrosis in healthy livers or exacerbate either feature in the setting of existing liver disease.

ACKNOWLEDGEMENTS

This research was supported by a grant from the Public Health Agency of Canada. Ms M. Labine is a recipient of a Manitoba Health Research Council fellowship award. The authors would also like to thank Ms R. Vizniak for her prompt and accurate typing of the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

There was no conflict of interest. Approval for the study was obtained from the University of Manitoba animal ethics committee. Funding was received from a graduate studentship from Manitoba Water Stewardship for Meaghan Labine.

REFERENCES

1. Duy TN, Lam PK, Shaw GR, Connell DW. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. *Rev Environ Contam Toxicol* 2000; 163: 113-85.
2. Carmichael WW. Health Effects of Toxin-Producing Cyanobacteria: The CyanoHABs. Human and Ecological Risk Assessment. *An International Journal* 2001; 7: 1393-407.
3. Pouria S, de Andrade A, Barbosa J, Cavalcanti RL, Barreto VT, Ward CJ, Preiser W, et al. Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet* 1998; 352: 21-6.
4. Yuan M, Carmichael WW, Hilborn ED. Microcystin analysis in human sera and liver from human fatalities in Caruaru, Brazil 1996. *Toxicon* 2006; 48: 627-40.
5. Falconer IR, Burch MD, Steffensen DA, Choice M, Coverdale OR. Toxicity of the blue green alga (cyanobacterium) Microcystis aeruginosa in drinking water to growing pigs, as an animal model for human injury and risk assessment. *Environ Toxicol Water Qual* 1994; 9: 131-9.
6. WHO. Algae and cyanobacteria in fresh water. In: Guidelines for safe recreational water environments. Vol 1. Coastal and fresh waters. Geneva, Switzerland: World Health Organization; 2003, p. 136-58.
7. Guzman RE, Solter PF. Hepatic oxidative stress following prolonged sublethal microcystin LR exposure. *Toxicol Pathol* 1999; 27: 582-8.
8. Runnegar M, Seward DJ, Ballatori N, Crawford JM, Boyer JL. Hepatic toxicity and persistence of ser/thr protein phosphatase inhibition by microcystin in the little skate Raja erinacea. *Toxicol Appl Pharmacol* 1999; 161: 40-9.

9. Malbrouck C, Trausch G, Devos P, Kestemont P. Effect of microcystin-LR on protein phosphatase activity and glycogen content in isolated hepatocytes of fed and fasted juvenile goldfish Carassius auratus L. *Toxicon* 2004; 44: 927-32.
10. Buratti FM, Scardala S, Funari E, Testai E. Human glutathione transferases catalyzing the conjugation of the hepatoxin microcystin-LR. *Chem Res Toxicol* 2011; 24: 926-33.
11. Carvalho GM, Oliveira VR, Casquilho NV, Araujo AC, Soares RM, Azevedo SM, Pires KM, et al. Pulmonary and hepatic injury after sub-chronic exposure to sublethal doses of microcystin-LR. *Toxicon* 2016; 112: 51-8.
12. He J, Li G, Chen J, Lin J, Zeng C, Chen J, Deng J, et al. Prolonged exposure to low-dose microcystin induces nonalcoholic steatohepatitis in mice: a systems toxicology study. *Arch Toxicol* 2017; 91: 465-80.
13. Elleman TC, Falconer IR, Jackson AR, Runnegar MT. Isolation, characterization and pathology of the toxin from a Microcystis aeruginosa (= Anacystis cyanea) bloom. *Aust J Biol Sci* 1978; 31: 209-18.
14. Frangez R, Kosec M, Sedmak B, Beravs K, Demsar F, Juntes P, Pogacnik M, et al. Subchronic liver injuries caused by microcystins. *Pflugers Arch* 2000; 440: R103-104.
15. Milutinovic A, Zorc-Pleskovic R, Petrovic D, Zorc M, Suput D. Microcystin-LR induces alterations in heart muscle. *Folia Biol (Praha)* 2006; 52: 116-8.

Correspondence and reprint request:
G.Y. Minuk, M.D.
Morberg Family Chair in Hepatology
University of Manitoba.
John Buhler Research Centre 715 McDermot Ave.
Winnipeg, MB R3E 3P4
Phone: (204) 789-3204. Fax: (204) 789-3987
E-mail: Gerald.Minuk@umanitoba.ca